ULTRAFILTRATION OF RECENTLY ISOLATED NEUROTROPIC VIRUSES

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Among the several filtrable agents encountered in Africa and South America by Rockefeller Foundation staff members and their co-workers, 11 have proved to be separate and distinct viral entities (Smithburn, 1952; Kerr, 1952). Since early in 1949 a number of workers have been engaged in systematic investigation of these agents. The present paper reports the studies of their size by means of ultrafiltration.

The origins of all the agents included in the study have been listed in previous reports (Smithburn, 1952; Dick and Haddow, 1952; Dick *et al.*, 1952). The viruses are the following: Semliki forest and Kumba (East and West African strains which appear to be identical), Bwamba fever, West Nile, Bunyamwera, Ntaya, Zika, Uganda S, Anopheles A, Anopheles B, Wyeomyia, Ilhéus, and Mengo. The last is identical with encephalomyocarditis virus, the size of which had been investigated previously by ultrafiltration (Warren, 1952). Inclusion of the Mengo strain in the present study afforded a comparison of our results with those of others obtained with collodion membranes prepared and calibrated in a different manner. The collodion membranes used in the present experiments were prepared and calibrated by the methods described by Bugher (1953).

METHODS

Fresh mouse brain passage virus was used in all the experiments. Source groups of mice were inoculated expressly for this usage, and the animals were sacrificed soon after the onset of objective illness. Six or seven brains from sick or paralyzed mice were milled in an aerosol-proof blendor (with overhead drive), using 5 per cent nonimmune rhesus monkey serum in 0.85 per cent sodium chloride to make a $10^{-1.5}$ suspension. This original suspension was spun either at 2,000 rpm in the horizontal centrifuge or at 7,000 rpm in the angle centrifuge; the supernate was subject to one or more preliminary filtrations either through a Seitz EK pad or through membranes having pore diameters of 600 to 800 mμ. The filter clarified supernate was distributed then in 5 to 6 ml quantities to chambers fitted with graded membranes. Filtration was allowed to proceed at room temperature under 20 to 40 pounds nitrogen pressure. If filtrations proceeded very slowly, they were stopped after 3 to 4 ml had passed through the membranes, but usually the entire amount was allowed to pass through. When all the filtrations were finished, the undiluted filtrates were tested separately by intracerebral inoculation into groups of 12 to 18 susceptible young adult Swiss mice. Portions of the original unfiltered supernate and of all preliminary filtrates were kept refrigerated during the filtration through graded membranes; on completion of the latter, dilutions were prepared and these materials were titrated intracerebrally. The titrations determined the potency of the original preparation and of the material distributed to the graded filters.

Filtrates through the graded membranes were regarded as containing virus if 2 of 12 or 3 of 18 inoculated mice exhibited signs of specific illness and succumbed within the appropriate period for the virus concerned.

With each virus one or two preliminary experiments were performed using single graded membranes of six to nine different pore diameters covering a wide range, in order to make a crude estimate of the end point. When this information was at hand, two to six additional experiments were performed, each employing duplicate or triplicate membranes with a narrower range of pore diameters anticipated to embrace the end point.

RESULTS

A few general observations made in the course of the experiments seem worthy of brief mention at this point.

The Seitz EK pads employed in some of the preliminary clarifications were the prewar product of the American Seitz Corporation. Immediately before use in these experiments they were in-

variably washed with a liberal quantity of serum-saline. Nevertheless, the titrations showed that passage of most of the viruses through Seitz pads was associated with significant loss of potency, the decline ranging from 0.5 to 2.0 in logarithmic titer.

By contrast with this, it was found that passage through collodion membranes having pore diameters of approximately 800 mμ was associated with no loss of potency at all. However, in order to accomplish the filtration through such a membrane, it was necessary either that the original $10^{-1.5}$ suspension first be filtered through a Seitz pad, or else be spun for at least 20 minutes at 7,000 rpm before filtration. Low speed centrifugation without Seitz filtration left the suspension insufficiently cleared to prevent obstruction of the pores of collodion membranes of 800 mμ APD.

In a few experiments viruses failed to pass through membranes having pore diameters well above the end point ultimately determined. It is believed that these results were due to aggregation of particles from causes unknown. The fact that such events can occur requires that the investigator avoid hasty judgment on the end point determination.

When the experiments were first undertaken, it was not known precisely what degree of variability in pore diameter should be accepted as representative of a reliable membrane. In the earlier screening experiments membranes with both low and high degrees of variation were employed and there was a considerable incidence of discordant results. An index of calibration variability, designated "*k* value", was devised by Bugher (1953). For the final experiments with all the viruses, only membranes with *k* values of 0.07 or less were used, and discordant results were encountered much less commonly.

In order to illustrate the differences in results obtained with membranes of low and high calibration variability, the filtration data are summarized in table 1. All results precisely *in* the end point range are omitted because of the in-

TABLE 1

Variations in results of filtrations dependent on uniformity of membranes

	MEMBRANES WITH $k \leq 0.07$	"ERROR"	MEMBRANES WITH $k > 0.07$	"ERROR"
		%		%
APD above* end point†				
Number tested	232		56	
Number negative	32		17	
APD below* end point†				
Number tested	148		62	
Number positive	8		12	
All membranes†				
Number tested	380		118	
"Errors"	40	10.5	29	24.6
Calculated error frequency				
k (mean)	0.05		0.11	
u‡	0.08		0.14	
Total error		11.0		20.0

* Membranes with APD between 140 and 170 mμ in experiments with large viruses and those with APD between 45 and 61 mμ in experiments with small viruses were omitted from this tabulation as, being in the end point range, they were especially subject to variable results.

† Tests with Wyeomyia virus were omitted because some experiments failed owing to weak virus preparations. Experiments with Mengo virus were not included because the range of membranes was different.

‡ *u* is the fraction above and below the mean APD which defines the end point range. Considering as "errors" observations falling outside this range, their expected frequency may be computed by the method cited (Bugher, 1953).

herent variability characteristic of the cut-off zone. In a total of 498 individual filtrations, the percentage of results contrary to expectation with membranes of low k value was less than half the rate with membranes of high k value. It is evident, therefore, that although this results in rejection of a considerable portion of the membranes which may be made, the final end point filtrations should be done only with membranes having low indexes of variability in pore diameter.

with the several viruses were quite different. There was a tendency of the agents to fall into two groups—one large, with the end point approximating 150 mμ, the other small, with the end point at 45 to 61 mμ. The Mengo virus did not fall into either of these groups as it and its prototypes (Warren, 1952) are apparently smaller than any of the other agents included in this study.

Results obtained with membranes having high calibration variability have been rejected. Data

TABLE 2

Results of filtration experiments with membranes of low calibration variability: Viruses of large particle size

VIRUS AND NO. EXPERIMENTS		APD, mμ						
		297-249	224-193	170	163	151	140	121-42
Anopheles A (6)	No. tests	5	21	4	1	5	4	4
	Positive	5	19	1	0	0	0	0
	Negative	0	2	3	1	5	4	4
Anopheles B (6)	No. tests	4	14	5	1	3	5	6
	Positive	4	14	3	0	0	0	0
	Negative	0	0	2	1	3	5	6
Wyeomyia (6)	No. tests	8	21	7	1	2	3	13
	Positive	6	7	2	0	0	0	1
	Negative	2	14	5	1	2	3	12
Ntaya (9)	No. tests	1	9	7		3	10	50
	Positive	1	9	6		0	1	4
	Negative	0	0	1		3	9	46
Bwamba fever (4)	No. tests	3	11	5	3	5	1	1
	Positive	3	10	3	2	0	0	0
	Negative	0	1	2	1	5	1	1
Bunyamwera (7)	No. tests	1	8	8		3	12	22
	Positive	1	8	8		3	2	1
	Negative	0	0	0		0	10	21

Filtrates from membranes with pore diameters well above the end point almost invariably caused 100 per cent mortality in inoculated mice. By contrast, filtrates from membranes just above the end point frequently caused death of only a part of the mice receiving them. Furthermore, membranes very near the end point might in one test withhold all the virus and in another allow some to pass, whereas membranes with much larger pores regularly allowed passage of the virus.

As was to be expected, the end points obtained from all other tests are shown in table 2 for the larger viruses and in table 3 for the smaller ones.

DISCUSSION

In tables 2 and 3 the end point for each virus is indicated by a double vertical line to the left of the limiting membrane. In the case of Anopheles A, the infective agent was recovered only once in four trials from filtrates through membranes of 170 mμ APD, so the virus may be even larger than the assigned end point would indicate.

From the data shown in tables 2 and 3, it may

TABLE 3

Results of filtration experiments with membranes of low calibration variability: Viruses of small particle size

VIRUS AND NO. EXPERIMENTS		APD, mμ										
		276-221	170-151	140-105	94-79	69	61	53	45-42	38	31	24-19
West Nile (5)	No. tests		4	10	10	9	7	5	3			
	Positive		3	9	6	3	0	0	0			
	Negative		1	1	4	6	7	5	3			
Semliki forest (5)	No. tests		4	7	7	5	5	6	7			
	Positive		4	6	6	5	1	1	0			
	Negative		0	1	1	0	4	5	7			
Kumba (5)	No. tests	2	2	9	7	3	3	3	6			
	Positive	2	2	8	4	3	2	0	0			
	Negative	0	0	1	3	0	1	3	6			
Zika (6)	No. tests	6	1	5	4	4	4	4	7			
	Positive	6	1	5	4	2	4	0	0			
	Negative	0	0	0	0	2	0	4	7			
Ilhéus (3)	No. tests	1		4	3	4	4	4	4			
	Positive	1		4	3	4	3	0	0			
	Negative	0		0	0	0	1	4	4			
Uganda S (5)	No. tests	4		7	3	1	4	4	6			
	Positive	4		6	3	1	4	2	0			
	Negative	0		1	0	0	0	2	6			
Mengo (5)	No. tests	1	1	6	2	3	4	4	4	6	6	9
	Positive	1	1	6	2	3	4	4	3	4	0	1
	Negative	0	0	0	0	0	0	0	1	2	6	8

TABLE 4

Size of viruses according to scheme of Elford

VIRUS	PARTICLE SIZE, mμ
Anopheles A	81 to 122
Anopheles B	81 to 122
Wyeomyia	81 to 122
Ntaya	81 (75) to 122* (113)
Bwamba fever	75 to 113
Bunyamwera	70 to 105
West Nile	20 to 30
Semliki forest	20 to 30
Kumba	18 to 26
Zika	18 to 26
Ilhéus	18 to 26
Uganda S	15 to 22
Mengo	10 to 15

* No membranes available between 151 and 170 mμ, so end point may be inexact.

be seen that Wyeomyia, Ntaya, Bunyamwera, Semliki, and Mengo viruses were each recovered one or more times from filtrates in the range below the end point shown. In each instance the preponderance of results supports the end point assigned. The occasional recovery of virus below the selected point may be indicative of variability in particle size with occasional particles smaller than average; unusual tendency of the particles to aggregate so that only dispersed particles give a true end point; faulty membranes or other technical errors; or variability in the APD of the membranes. It was surprising, in fact, that the end point for Ntaya virus was so high in view of its demonstrated antigenic relationship to other viruses of smaller size (Smithburn, 1952; Kerr, 1952). It is possible that further study may reveal that certain of the agents have in their population at least some particles smaller than present findings indicate.

It will be noted that the end points obtained with Semliki forest and Kumba viruses are not quite identical although these agents are reciprocally cross-reactive (Smithburn, 1952; Kerr, 1952) and apparently identical immunologically. No reason can be assigned at present for their slightly differing end points in the filtration experiments.

The end point obtained for Mengo virus is within the range stated by Warren (1952) for encephalomyocarditis virus, showing not only that this agent is smaller than the other viruses in the study, but that the results obtained by the methods here described are comparable to those of other workers.

The end point here obtained for Bwamba fever virus is somewhat lower than was obtained previously by Smithburn *et al.* (1941), and the end point for Uganda S is lower than was found by Dick and Haddow (1952). The results with West Nile virus are in good agreement with those reported by Smithburn *et al.* (1940).

According to the standards adopted by Elford (1933) for the interpretation of filtration end points, the results obtained indicate the approximate particle sizes of the viruses studied to be as shown in table 4.

SUMMARY

Eleven distinct and different recently discovered neurotropic viruses have been studied by filtration through graded collodion membranes. The agents fall into two groups according to size. The larger, with filtration end points approximating 150 mμ, includes Anopheles A, Anopheles B, Wyeomyia, Ntaya, Bwamba fever, and Bunyamwera viruses. The group of smaller viruses includes West Nile, Semliki forest, Zika, Ilhéus, and Uganda S, each having filtration end points in the 45 to 61 mμ range.

The calibration of five or more disks per sheet and the rejection of all lots showing high calibration variability, as proposed by Bugher, ensure that the error of the method of ultrafiltration will be reduced appreciably.

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